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ORIGINAL PAPER

Synthesis of fatty acid esters and diacylglycerols at elevated temperatures by alkalithermophilic lipases from *Thermosyntropha lipolytica*

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Abstract LipA and LipB of *Thermosyntropha lipolytica* DSM 11003 as previously published are the most alkalithermophilic ($pH_{opt}^{25°C} = 9.4–9.6$, $T_{opt} = 96°C$) and thermostable ($T_{1/2}^{24\ h} = 74–76°C$) lipases currently known. The purified enzymes were analyzed in organic solvents for their ability to catalyze synthesis of diacylglycerols and various alcohol fatty acids. To obtain 100% recovery and avoid a 40% and 50% loss of catalytic activity during lyophilization of purified LipA and LipB, respectively, addition of 1 mg/ml bovine serum albumin (BSA) and 25% polyethylene glycol (PEG400) was required. LipA and LipB catalyzed esterification of fatty acids and alcohols with the highest yields for octyl oleate (LipA) and lauryl oleate (LipB) and also catalyzed synthesis of 1,3-dioleoyl glycerol, 1-oleoyl-3-lauroyl glycerol, and 1-oleoyl-3-octoyl glycerol. Isooctane was the most efficient solvent for esterification reactions at 85°C. Similar to the positional specificity for the hydrolytic reaction in aqueous solutions, LipA and LipB catalyzed in organic solvents the synthesis of diacylglycerol with esterification of position 1 and 3 with a yield of 62% for di-oleoyl glycerol. The reported conversion rates do not represent the full potential of these enzymes, since only 1/100th–1/1,000th of the protein concentrations usually used in commercial processes were available. However, use of slightly increased protein concentrations confirmed the trend to higher yields with higher protein concentrations. The obtained specificity and variety of the reactions catalyzed by LipA and LipB, and their high thermostability allowing synthesis to occur at 90°C, demonstrate their great potentials for industrial applications, particularly in structured lipid biosynthesis for substrates that are less soluble at mesobiotic temperatures.

Keywords *Thermosyntropha lipolytica* · Alkalithermophilic · Lipase · Organic synthesis · Fatty acid esters · 1,3-Diacylglycerol

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Introduction

Thermosyntropha lipolytica, a thermophilic, alkalitolerant, lipolytic, syntrophic anaerobic bacterium [44], produces two commercially viable lipases for use at elevated temperatures and under alkaline conditions such as those used for laundering detergents [36, 39]. This thermophilic anaerobe grows syntrophically with hydrogen utilizers on triacylglycerols and ferments saturated and unsaturated fatty acids but not the glycerol [44]. LipA and LipB are true lipases (carboxyl ester hydrolases, E.C. 3.1.1.3) catalyzing the hydrolysis (as published elsewhere [37]) and synthesis (as shown herein) of long-chain fatty acid esters in nonaqueous milieu. Besides being used in laundry detergents, biodiesel production, lubricants, cosmetic formulations, and flavor and aroma constituents [6, 7, 20, 22, 23, 36, 41], lipases are increasingly used to catalyze enantioselective reactions for the synthesis of fine chemicals and the kinetic resolution of racemates [33]. A wide variety of alcohol fatty acids are used in manufacturing soaps and detergents, biodegradable replacements for

mineral oil, cosmetics, wood preservatives, and personal care products [16, 21, 25, 34].

One specific interest is the synthesis of diacylglycerols (DAG), which have multifunctional and nutritional properties. They are constituents of edible fats and oil but with fewer calories and similar digestibility to triacylglycerols (TAG). A diet containing DAG, especially *sn*-1,3-diacylglycerols, was found to reduce obesity, reduce total fat content in men, and enhance loss of body weight [28, 30, 31, 49]. As a result, 1,3-diacylglycerols were introduced in Japan as cooking oil under the trade name of Econa to reduce body fat accumulation [27, 28].

Thermophilic enzymes have several advantages over mesophilic ones, including higher stability and greater resistance to denaturation in organic solvents [12, 15, 39]; moreover, operating at high temperature will minimize problems associated with the solubility of some substrates [4, 46]. Examples of thermostability in organic solvents include porcine pancreatic lipase, which withstands heating at 100°C for many hours in an organic solvent [50]. Likewise, the thermal stability of a ribonuclease was greatly enhanced in organic solvent (T_m values as high as 124°C) compared with in aqueous solution (T_m value of 61°C) [45].

Previously, we reported on the purification and characterization of the two most thermophilic lipases, LipA and LipB, from an anaerobic thermophile *T. lipolytica*, with maximum catalytic activity in aqueous solution at 96°C (20 min assay) and optimum $pH^{80°C}$ of around 9.5 [37]. Both lipases show high thermostability, retaining 50% of their activity after 24 h incubation at 75°C, and are stable at room temperature in the presence of 0.5 M ammonium sulfate at neutral and alkaline $pH^{25°C}$ ($pH^{25°C}$ 7.0–12.0) without loss of activity for at least 4 weeks. Maximum catalysis of hydrolysis reactions was observed with glycerides containing long-chain fatty acids [37]. This report describes the stability and activity of these two lipases in nonaqueous solutions at elevated temperatures and—in a proof of concept—shows the esterification of oleic acid and other long-chain fatty acids with various alcohols in organic solvents at temperatures as high as 90°C. The described reactions open up this process for some substrates not soluble at or reactive enough at mesobiotic temperatures.

Materials and methods

Culture conditions

T. lipolytica (DSMZ 11003) was grown in a basal medium containing 0.75% yeast extract as carbon and energy source under nitrogen gas phase as described in a previous report [37]. The pH of the medium was adjusted to $8.2^{25°C}$ and the growth temperature was 60°C.

Lipase assay

Lipase was assayed by two methods. The first method used the chromogenic substrate *p*-nitrophenyl laurate (*p*NPL) (Sigma, USA) [37]. One unit was defined as the amount of enzyme catalyzing the release of 1 μmol *p*-nitrophenol per min from *p*NPL. The second method employed the NEFA C kit (Waco, USA). This assay, frequently used in industrial settings, is a sensitive method for measuring nonesterified free fatty acids (NEFA) and could be effectively used to quantify the depletion of NEFA and consequently the formation of esterified fatty acids. Briefly, this assay relies on the acylation of coenzyme A by the free fatty acids in the presence of added acyl-CoA synthetase. The produced acyl-CoA is oxidized by acyl-CoA oxidase with the generation of hydrogen peroxide, which in the presence of peroxidase, permits the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(*β*-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple color that is measured spectrophotometrically at 550 nm. For this assay, one unit was defined as releasing 1 μmol free fatty acid from glycerides per minute.

Purification of lipases for synthesis in organic solutions

After 18 h of growth in a 20-l fermentor, the cells of *T. lipolytica* were removed from the culture broth using the 1 million cutoff Amicon hollow fiber filtration system (Millipore). The proteins in the supernatant were then concentrated by filtration through a 10-kDa Amicon hollow fiber system and subsequently precipitated by slow addition of four volumes of cold (−20°C) acetone. The white precipitate was collected by centrifugation and then dissolved in 20 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer, pH 9.0. The protein solution was then loaded onto an Octyl Sepharose fast flow column (Amersham Biosciences) (30 ml bed volume), and the two lipases, LipA and LipB, were eluted separately at 1.8 and 0.5 M ammonium sulfate in Tris buffer pH 8.0, respectively [37]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing PAGE were performed essentially as described by Ausubel [5] and Sambrook [38]. Gels were stained with GelCode® Blue Stain Reagent (Pierce). Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay kit (Pierce).

Enzyme lyophilization

Aliquots (100 or 50 μg) of LipA and LipB were lyophilized in 2-ml serum bottles. Prior to lyophilization, protein

samples were dialyzed against 100 mM TAPS buffer (pH 9.0) containing 1 mM $MnCl_2$, followed by addition of 25% polyethylene glycol (PEG400) (v/v) and 1 mg/ml bovine serum albumin (BSA) to reach a final volume of 0.4 ml.

Synthesis of fatty acid esters and diacylglycerols (DAG)

The esterification reactions were carried out in closed vials containing usually 100 μg lyophilized enzyme (50 μg/ml final concentration) unless otherwise stated. The esterification mixtures for the synthesis of alcohol esters (Fig. 1a) were composed of 100 mM fatty acids and 100 mM alcohols in 2 ml isooctane, whereas the diacetylglycerols synthesis (Fig. 1b) solution contained 100 mM fatty acids and 100 mM monoacylglycerols. Reactions were incubated at 85°C under shaking (300 rpm) for up to 100 h. Quantitative analysis was done by measuring the remaining unesterified free fatty acids by using NEFA C kit. Synthesis products were verified by thin-layer chromatography.

Effect of temperature on activity and stability in isooctane

The effect of temperature on activity was investigated by determining the esterification of 1-heptanol, 1-octanol, 1-dodecanol, and oleic acid in isooctane. The esterification reaction was carried out in isooctane (2 ml) containing 50 mM of the alcohol and 50 mM oleic acid with 50 μg lyophilized enzyme. The vials were incubated at the indicated temperatures with shaking at 300 rpm for 60 h. Aliquots for TLC analysis were withdrawn at periodic intervals. For quantitative measurements, samples were withdrawn, concentrated by evaporation, and then used to quantify the nonesterified fatty acids (NEFA) by using NEFA C kit. To estimate LipA and LipB stability in isooctane, 100 μg lyophilized LipA and LipB were suspended in 1 ml isooctane in sealed 5-ml serum bottles and incubated at 85°C; 100 μl aliquots were withdrawn at each time point, lyophilized as described above, and assayed for enzymatic activities using the chromogenic substrate *p*-nitrophenyl laurate.

Thin-layer chromatography (TLC)

At various time points, aliquots of the esterification reaction mixture were applied on reversed phase (Multi-K) TLC plates (Whatman, USA) to assay for DAG and fatty acid esters (butyl oleate, heptyl oleate, octyl oleate, nonanyl oleate, and lauryl oleate). The plates were developed first with a mixture of petroleum:diethyl ether:acetic acid (70:30:1) until the solvent front reached half of the plate, then continued after drying with chloroform:acetone:acetic acid (96:4:1). When the second solvent reached the end of the plate, spots were visualized by spraying the dried plates with iodine vapor (0.1% iodine in chloroform).

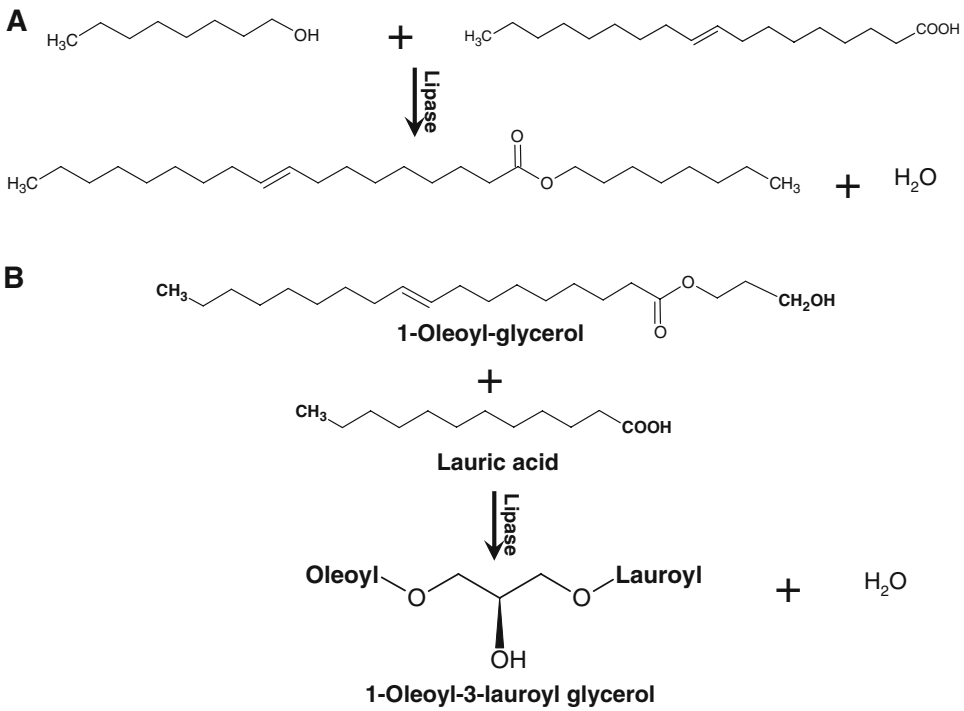

Fig. 1 Esterification reactions conducted in this study: synthesis of (**a**) the fatty acid ester, octyl oleate, and (**b**) the diacylglycerol, 1-oleoyl-3-lauroyl glycerol

Results and discussion

Lyophilization of purified lipases

The two extracellular lipases LipA and LipB were purified to gel-electrophoretic homogeneity (Fig. 2) from *T. lipolytica* culture supernatant as described in the "Materials and methods" by acetone precipitation and hydrophobic interaction chromatography, a simplified procedure compared with that originally published [37]. The purification yield was 42% for both enzymes, combined with a purification factor of 50, and a final specific activity of 12.0 and 12.8 U/mg for LipA and LipB, respectively. $pH^{25°C}$ was adjusted to the $pH^{25°C}_{optimum}$ before lyophilization. This was done to take advantage of the proteins' "pH memory;" that is, their ionization and thus their catalytic activity in organic solvents is a reflection of the pH of the last aqueous solution to which they were exposed [52]. Addition of 1 mg/ml bovine serum albumin (BSA) and 25% polyethylene glycol (PEG400) (v/v) as cryoprotectant [13] prior to lyophilization was necessary to prevent the otherwise observed 40% and 50% inactivation of purified LipA and LipB, respectively, which is similar to the effect observed by Griebenow and Klibanov [18]. Furthermore, these additions lead to approximately five times higher esterification rates. PEG was not used as an acyl acceptor by LipA or LipB. When the crude protein solutions obtained by the acetone precipitation were used, lyophilization did not cause a loss of activity, presumably due to the presence of noncatalytic proteins in the culture supernatant acting as cryoprotectants and keeping the enzymes in their enzymatically active conformation [14].

It is important to point out that the presence of extreme low water activity in organic solvents is fundamentally important in order to shift the reaction effectively toward synthesis rather than hydrolysis [3, 10, 19, 52].

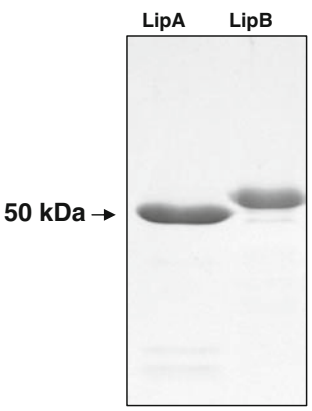

Fig. 2 SDS-PAGE analysis of the purified LipA and LipB

Selection of the optimal organic solvent

Because the type of organic solvent affects the thermostability [42], rates of esterification and condensation reactions [1, 11, 35, 47, 48], and the enantioselectivity [9, 17, 29, 32, 50, 51], several solvents were tested. Isooctane was the most effective solvent for synthesis of fatty acid esters. Using 100 μg enzyme (50 μg/ml) and 60 h of incubation, LipA catalyzed esterification of oleic and lauryl acid with octanol, yielding 25% octyl oleate and 21% lauryl oleate. LipB showed similar efficiency in isooctane; however, lauryl oleate synthesis was slightly favored (Table 1). As expected, the more hydrophilic acetonitrile was a poor solvent for organic synthesis [3, 8, 24, 45].

Synthesis of fatty acid esters at various temperatures

The maximum temperature for catalytic activity to synthesize fatty acid esters in organic solvents was determined by conducting esterification of oleic acid with octanol in isooctane at different temperatures. Similar to reactions in aqueous solution, LipA and LipB had a maximum activity at 85–90°C in isooctane (Fig. 3a). At 85°C, both lipases lost about 35% and 75% of their activity after 24 and 96 h, respectively, indicating half lives of about 48 h at 85°C in isooctane (Fig. 3b). Although both enzymes showed lower catalytic activity than we reported previously in aqueous medium [37], the enzymes in fact showed a dramatic increase in stability in isooctane compared with the stability observed in aqueous environments [37]. Such an effect observed with other lipases has been attributed to structural rigidity [19].

Both enzymes, LipA and LipB, catalyzed esterification of oleic acid with all tested intermediate and long-chain alcohols, including 1-heptanol, 1-octanol, 1-nonanol, and 1-dodecanol, but did not react with 1-butanol (Fig. 4). Using the nonindustrial low enzyme concentration of 50 μg/ml, the highest conversion was achieved by LipA, yielding 25% octyl oleate and 23% lauryl oleate. The maximum conversion by LipB was 24% of lauryl oleate and 21% of octyl oleate. The lipase-catalyzed synthesis was monitored by TLC throughout the reaction time course.

As previously reported, both LipA and LipB exhibited maximum hydrolysis of lipids with long-chain unsaturated fatty acids followed by saturated long-chain fatty acids but not with lipids containing short (<8 carbon) fatty acids [37]. A similar correlation was also observed in the reverse direction, i.e., ester synthesis. Oleic acid was the preferred acyl donor in the esterification reactions, followed by the saturated palmitic acid, lauric acid, and butyric acid. After 60 h at 85°C, 25% conversion to octyl-oleate, 8% octyl palmitate and octyl laurate, and 0% octyl butyrate was obtained. The relative low conversion rate observed is

Table 1 Lipase-catalyzed esterification of fatty acid with alcohols in various organic solvents

Lipase	Solvent	Reaction temperature (°C)	Octyl oleate[b] Conversion (%)	Lauryl oleate[b] Conversion (%)	Octyl oleate[c] Conversion (%)	Lauryl oleate[c] Conversion (%)
LipA	Isooctane	85	14 ± 1.8	8 ± 1.0	25 ± 2.2	21 ± 2.0
	Octane	85	11 ± 0.9	6 ± 0.9	22 ± 1.2	20 ± 2.0
	Toluene	85	6 ± 0.5	3 ± 0.8	11 ± 0.4	10 ± 0.7
	Acetonitrile	75	1 ± 0.4	2 ± 0.4	1 ± 0.3	1 ± 0.1
LipB	Isooctane	85	12 ± 2.0	9 ± 1.2	23 ± 1.7	24 ± 0.9
	Octane	85	12 ± 1.6	8 ± 1.6	22 ± 1.3	21 ± 0.2
	Toluene	85	10 ± 3.1	8 ± 2.2	15 ± 3.0	18 ± 1.5
	Acetonitrile	75	0	0	2 ± 0.2	0
SN[a]	Isooctane	85	19 ± 0.9	18 ± 1.1	34 ± 2.4	30 ± 2.0
	Octane	85	20 ± 1.3	18 ± 1.6	37 ± 1.9	32 ± 2.0
	Toluene	85	10 ± 3.6	8 ± 3.0	14 ± 3.3	11 ± 2.2
	Acetonitrile	75	3 ± 0.6	2 ± 0.1	5 ± 1.0	5 ± 0.6

[a] SN is supernatant concentrate after acetone precipitation (~0.4 mg protein, Sp. act 5 U/mg)

[b] Reaction time was 12 h

[c] Reaction time was 60 h

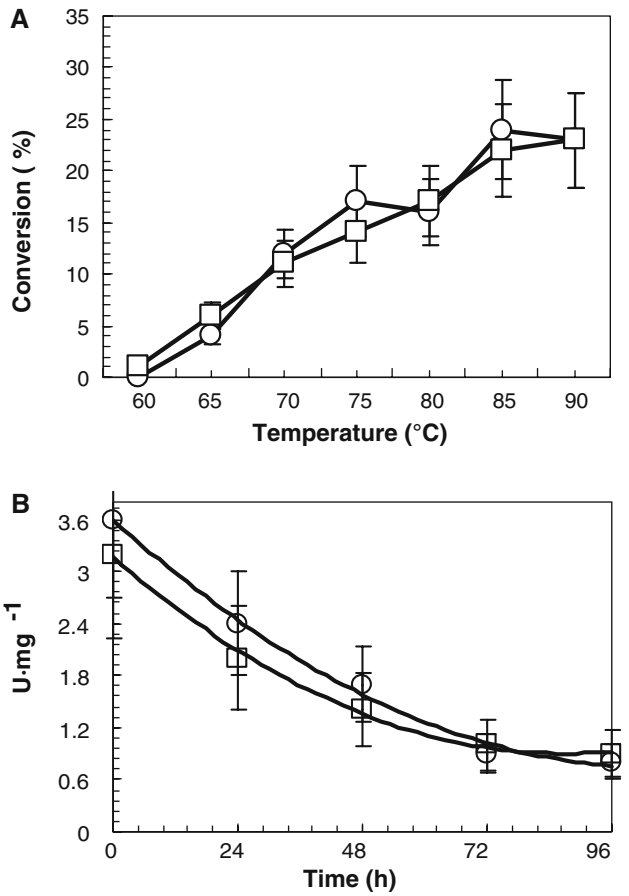

Fig. 3 Effect of temperature on LipA (*open circles*) and LipB (*open squares*) activity (**a**) and stability (**b**) in isooctane. Optimum catalytic activity and stability were determined as described in the "Materials and methods". An enzyme sample in isooctane kept at 25°C was used as reference. A 100% relative activity was 3.6 and 3.3 U/mg for LipA and LipB, respectively

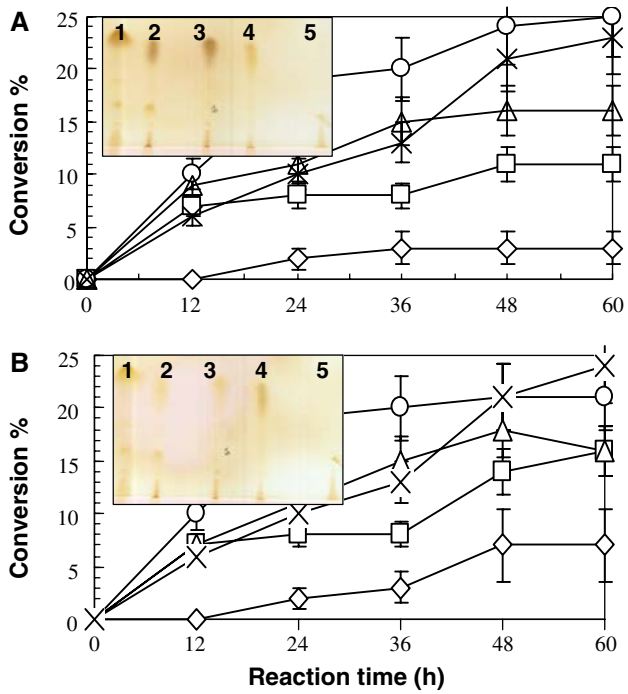

Fig. 4 Time course of fatty acid ester synthesis catalyzed by LipA (**a**) and LipB (**b**). Butyl oleate (*open diamonds*), heptyl oleate (*open squares*), octyl oleate (*open circles*), nonanyl oleate (*open triangles*), and lauryl oleate (*crosses*). The insert depicts TLC analysis of fatty acid esters after 60 h of reaction time. *Lanes* from left to right are lauryl oleate (*1*), nonanyl oleate (*2*), octyl oleate (*3*), heptyl oleate (*4*), and butyl oleate (*5*)

attributed to the low concentration of the lipases since heterologously expressed proteins to use milligram amounts of enzymes were not available. In industrial applications with high product yields, the applied protein

concentrations are usually 100- to 1,000-fold higher. To test this assumption, in one assay, i.e., esterification of octanol and oleic acid, the lipase concentrations were raised from 50 μg/ml assay to 125 μg LipB and 200 μg LipA per milliliter assay. This moderate increase in enzyme concentration led to product yield increase from about 25% to 48% and 58%, respectively, thus supporting our above assumption. Based on reports in the literature, we hypothesize that, in addition, improving the lipases through genetic alterations for specific applications, along with immobilization, will generate a process with industrially required efficiencies [10, 40, 42]. Crude acetone precipitation yielded higher conversion rates of up to 37%, indicating that crude enzyme precipitations are effective and could be used in industrial processes.

Diacylglycerol synthesis

The capability of LipA and LipB to synthesize effectively diacylglycerol (DAG) was demonstrated by using 1-oleoyl glycerol, 1-lauroyl glycerol, and various fatty acids as substrates (Fig. 5). Both lipases led to extensive esterification. 1,3-Di-oleoyl glycerol exhibited the highest conversion percentage (62%) among all of the DAGs generated. In contrast, synthesis of DAG from 1-lauroyl glycerol and various fatty acids was low. TLC analysis revealed no indication of transesterification or formation of any triacylglycerols or 1,2-acylglycerols from glycerol, i.e., no indications of esterification at the 2-position, thus exhibiting the same 1- and 3-position specificity of LipA and LipB during hydrolysis in aqueous solutions [37].

In conclusion, the presented data demonstrate the various capabilities of the two investigated lipases to synthesize, at elevated temperatures of up to 90°C, in organic solvents, and with position specificity at 1- and/or 3-position, various acylglycerides, as well as synthesis of various alcohol fatty acids. The positional specificity of LipA and LipB makes them attractive enzymes for technical applications, especially for synthesis of structured lipid, flavor and aroma constituents where elevated temperatures are used. However, concentrated recombinant enzyme preparations are needed to further achieve the industrially desired high conversion rates, i.e., to perform the reactions under industrial conditions, using 100- to 1,000-fold higher protein concentrations than were available in this proof-of-concept project [2, 26, 43].

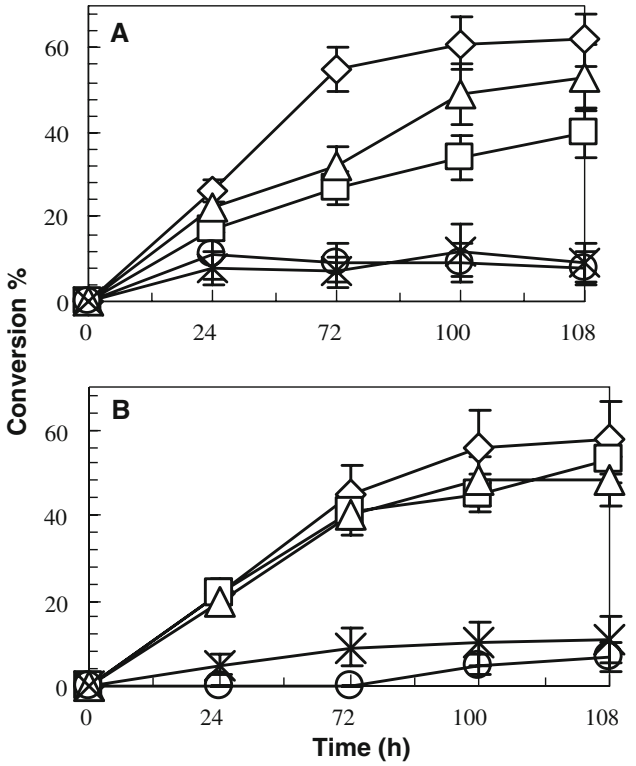

Fig. 5 Time course of diacylglycerol synthesis catalyzed by LipA (**a**) and LipB (**b**). 1,3-Dioleoyl glycerol (*open diamonds*), 1-oleoyl-3-lauroyl glycerol (*open squares*), 1-oleoyl-3-octoyl glycerol (*open triangles*), 1,3-dilauroyl glycerol (*crosses*), and 1-lauroyl-3-oleoyl glycerol (*open circles*)

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